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(71) Applicant: LUDWIG INSTITUTE FOR CANCER RESEARCH [CH/US]; 1345 Avenue of the Americas, New York, NY 10022 (US).			
(72) Inventors: VAN DER BRUGGEN, Pierre; Avenue Hippocrate 74, UCL 7459, B-1200 Brussels (BE). VAN DEN EYNDE, Benoit; Avenue Hippocrate 74, UCL 7459, B-1200 Brussels (BE). DeBACKER, Olivier; Avenue Hippocrate 74, UCL 7459, B-1200 Brussels (BE). BOON-FALLEUR, Thierry; Avenue Hippocrate 74, UCL 7459, B-1200 Brussels (BE).			
(74) Agent: HANSON, Norman, D.; Felfe & Lynch, 805 Third Avenue, New York, NY 10022 (US).			
(54) Title: ISOLATED, TRUNCATED NUCLEIC ACID MOLECULES WHICH CODE FOR GAGE TUMOR REJECTION ANTIGEN			
(57) Abstract			
<p>A new family of tumor rejection antigen precursors, and the nucleic acid molecules which code for them, are disclosed. These tumor rejection antigen precursors are referred to as GAGE tumor rejection antigen precursors, and the nucleic acid molecules which code for them are referred to as GAGE coding molecules. Various diagnostic and therapeutic uses of the coding sequences and the tumor rejection antigens, and their precursor molecules are described. Tumor rejection antigens are also shown.</p>			

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"ISOLATED, TRUNCATED NUCLEIC ACID MOLECULES WHICH CODE FOR GAGE
TUMOR REJECTION ANTIGEN"

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FELIPE & LYNN

NORMAN D. HANSON

1990-1991
Year

RELATED APPLICATION

This application is a continuation-in-part of copending application Serial No. 08/370,648, filed January 10, 1995, which is a continuation in part of copending patent application Serial No. 5 08/250,162 filed on May 27, 1994, which is a continuation-in-part of Serial No. 08/096,039 filed July 22, 1993. All of the applications are incorporated by reference.

FIELD OF THE INVENTION

10 This invention relates to a nucleic acid molecule which codes for a tumor rejection antigen precursor. More particularly, the invention concerns genes, whose tumor rejection antigen precursor is processed, inter alia, into at least one tumor rejection antigen that is presented by HLA-Cw6 molecules. The genes in question do not appear to be related to other known tumor rejection antigen 15 precursor coding sequences. The invention also relates to peptides presented by the HLA-Cw6 molecules, and uses thereof. Also a part of the inventions are peptides presented by HLA-A29 molecules, and uses thereof.

BACKGROUND AND PRIOR ART

20 The process by which the mammalian immune system recognizes and reacts to foreign or alien materials is a complex one. An important facet of the system is the T lymphocyte, or "T cell" response. This response requires that T cells recognize and interact with complexes of cell surface molecules, referred to as

human leukocyte antigens ("HLA"), or major histocompatibility complexes ("MHCs"), and peptides. The peptides are derived from larger molecules which are processed by the cells which also present the HLA/MHC molecule. See in this regard Male et al., 5 Advanced Immunology (J.P. Lipincott Company, 1987), especially chapters 6-10. The interaction of T cells and HLA/peptide complexes is restricted, requiring a T cell specific for a particular combination of an HLA molecule and a peptide. If a specific T cell is not present, there is no T cell response even 10 if its partner complex is present. Similarly, there is no response if the specific complex is absent, but the T cell is present. This mechanism is involved in the immune system's response to foreign materials, in autoimmune pathologies, and in responses to cellular abnormalities. Much work has focused on the mechanisms by which 15 proteins are processed into the HLA binding peptides. See, in this regard, Barinaga, *Science* 257: 880 (1992); Fremont et al., *Science* 257: 919 (1992); Matsumura et al., *Science* 257: 927 (1992); Latron et al., *Science* 257: 964 (1992). Also see Engelhard, *Ann. Rev. Immunol.* 12: 181-207 (1994).

20 The mechanism by which T cells recognize cellular abnormalities has also been implicated in cancer. For example, in PCT application PCT/US92/04354, filed May 22, 1992, published on November 26, 1992, and incorporated by reference, a family of genes is disclosed, which are processed into peptides which, in turn, are 25 expressed on cell surfaces, which can lead to lysis of the tumor cells by specific CTLs cytolytic T lymphocytes, or "CTLs"

h r after. The g nes are said to code for "tumor rejection antigen precursors" or "TRAP" molecules, and the peptides derived therefrom are referred to as "tumor rejection antigens" or "TRAs". See 5 Traversari et al., Immunogenetics 35: 145 (1992); van der Bruggen et al., Science 254: 1643 (1991), for further information on this family of genes. Also, see U.S. patent application Serial Numb r 807,043, filed December 12, 1991, now U.S. Patent No. 5,342,774.

In U.S. patent application Serial Number 938,334, now U.S. Patent No. 5,405,940, the disclosure of which is incorporated by 10 reference, it is explained that the MAGE-1 gene codes for a tumor rejection antigen precursor which is processed to nonapeptides which are presented by the HLA-A1 molecule. The reference teaches that given the known specificity of particular peptides for particular HLA molecules, one should expect a particular peptide 15 to bind to one HLA molecule, but not to others. This is important, because different individuals possess different HLA phenotypes. As a result, while identification of a particular peptide as being a partner for a specific HLA molecule has diagnostic and therapeutic ramifications, these are only relevant for individuals 20 with that particular HLA phenotype. There is a need for furth r work in the area, because cellular abnormalities are not restrict d to one particular HLA phenotype, and targeted therapy requires some knowledge of the phenotype of the abnormal cells at issue.

In U.S. Patent Application Serial Number 008,446, filed 25 January 22, 1993 and incorporated by reference, the fact that th MAGE-1 xpr ssion product is proc ss d t 'a s c nd TRA is

disclos d. This second TRA is pr sented by HLA-C clone 10 molecules. The disclosure shows that a given TRAP can yield a plurality of TRAs.

5 U.S. Patent Application Serial Number 994,928, filed December 22, 1992, and incorporated by reference herein teaches that tyrosinase, a molecule which is produced by some normal cells (e.g., melanocytes), is processed in tumor cells to yield peptides presented by HLA-A2 molecules.

10 In U.S. patent application Serial No. 08/032,978, filed March 18, 1993, and incorporated by reference in its entirety, a second TRA, not derived from tyrosinase is taught to be presented by HLA-A2 molecules. The TRA is derived from a TRAP, but is coded for by a non-MAGE gene. This disclosure shows that a particular HLA molecule may present TRAs derived from different sources.

15 In U.S. patent application Serial No. 08/079,110, filed June 17, 1993 and incorporated by reference herein, an unrelated tumor rejection antigen precursor, the so-called "BAGE" precursor is described. The BAGE precursor is not related to the MAGE family.

20 The work which is presented by the papers, patent, and patent applications cited supra deals, in large part, with the MAGE family of genes, and the unrelated BAGE gene. It has now been found, however, that additional tumor rejection antigen precursors are expressed by cells. These tumor rejection antigen precursors are referred to as "GAGE" tumor rejection antigen precursors. They do 25 not show homology to either the MAGE family of genes or the BAGE gene. Thus the present invention relates to genes enc ding such

TRAPs, the tumor rejection antigen precursors themselves as well as applications of both.

Thus, another feature of the invention are peptides which are anywhere from 9 to 16 amino acids long, and comprise the sequence:

5 Xaa_{1,2} Trp Xaa Xaa Xaa Xaa Xaa Tyr

(SEQ ID NO: 23)

where Xaa is any amino acid and Xaa_{0,1} means that 1 or 2 amino acids may be N-terminal to the Trp residue. These peptides bind to t₁ and/or are processed to peptides which bind to HLA-A29 molecules.

10 The invention is elaborated upon further in the disclosure
which follows.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 sets forth lysis studies using CTL clone 76/6.

Figure 2 shows tumor necrosis factor ("TNF") release assays obtained with various transfectants and controls.

Figure 3 compares lysis induced by cytolytic T lymphocytes of cl n CTL 76/6. Peptides of varying length were tested, including SEQ ID NO: 4.

Figure 4 presents an alignment of the cDNAs of the six GAGE genes discussed herein. In the figure, identical regions are surrounded by boxes. Translation initiation sites and stop codons are also

indicated. Primers, used in polymerase chain reaction as described in the examples, are indicated by arrows.

Figure 5 sets forth the alignment of deduced amino acid sequences for the members of the GAGE family. Identical regions are shown 5 by boxes, and the antigenic peptide of SEQ ID NO: 4, is shown.

Figure 6 shows the results obtained when each of the GAGE cDNAs was transfected into COS cells, together with HLA-Cw6 cDNA. Twenty-four hours later, samples of CTL 76/6 were added, and TNF release was measured after twenty-four hours.

10 Figure 7 compares the stimulation of CTL 22/23 by COS-7 CELLS, transfected with HLA-A29 cDNA, a MAGE, BAGE, or GAGE sequence, as shown. Control values are provided by MZ2-MEL.43 and COS cells, as stimulators.

15 Figure 8 presents results obtained from ^{51}Cr release studies, using various peptides including SEQ ID NO: 22 and various peptides derived therefrom.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Example 1

20 A melanoma cell line, MZ2-MEL was established from melanoma cells taken from patient MZ2, using standard methodologies. This cell line is described, e.g., in PCT Application PCT/US92/04354,

5 filed May 22, 1992, published November 26, 1992, and incorporated by reference in its entirety. Once the cell line was established, a sample thereof was irradiated, so as to render it non-proliferative. These irradiated cells were then used to isolate cytolytic T cell clones ("CTLs") specific thereto.

10 A sample of peripheral blood mononuclear cells ("PBMCS") was taken from patient MZ2, and contacted to the irradiated melanoma cells. The mixture was observed for lysis of the melanoma cells, which indicated that CTLs specific for a complex of peptide and HLA molecule presented by the melanoma cells were present in the sample.

15 The lysis assay employed was a chromium release assay following Herin *et al.*, Int. J. Cancer 39:390-396 (1987), the disclosure of which is incorporated by reference. The assay, however, is described herein. The target melanoma cells were grown in *vitro*, and then resuspended at 10^7 cells/ml in DMEM, supplemented with 10 mM HEPES and 30% FCS, and incubated for 45 minutes at 37°C with 200 μ Ci/ml of Na(⁵¹Cr)O₄. Labelled cells were washed three times with DMEM, supplemented with 10 mM Hepes. These 20 were then resuspended in DMEM supplemented with 10 mM Hepes and 10% FCS, after which 100 ul aliquots containing 10^3 cells, were distributed into 96 well microplates. Samples of PBLs were added in 100 ul of the same medium, and assays were carried out in duplicate. Plates were centrifuged for 4 minutes at 100g, and 25 incubated for four hours at 37°C in a 8% CO₂ atmosphere.

Plates were centrifuged again, and 100 ul aliquots of supernatant were collected and counted. Percentage of ^{51}Cr release was calculated as follows:

5

$$\% \text{ }^{51}\text{Cr} \text{ release} = \frac{(\text{ER}-\text{SR})}{(\text{MR}-\text{SR})} \times 100$$

where ER is observed, experimental ^{51}Cr release, SR is spontaneous release measured by incubating 10^3 labeled cells in 200 ul of medium alone, and MR is maximum release, obtained by adding 100 ul 0.3% Triton X-100 to target cells.

10

Those mononuclear blood samples which showed high CTL activity were expanded and cloned via limiting dilution, and were screened again, using the same methodology. The CTL clone MZ2-CTL 76/6 was thus isolated. The clone is referred to as "76/6" hereafter.

15

The same method was used to test target K562 cells, as well as the melanoma cell line. Figure 1 shows that this CTL clone recognizes and lyses the melanoma cell line, i.e. MZ2-MEL but not K562. The clone was then tested against other melanoma cell lines and autologous EBV-transformed B cells in the same manner described supra. Figure 1 shows that autologous B cells, transformed by Epstein Barr Virus ("EBV") were not lysed, and that while MZ2-MEL 3.0 was lysed by CTL clone 76/6, the cell line MZ2-MEL.4F, a variant which does not express antigen F, was not. Hence, the clone appears to be specific for this antigen.

20

The results presented supra are inconclusive as to which HLA molecule presents the TRA. The lysed cell line, i.e., MZ2-MEL, is

known to express HLA-A1, HLA-A29, HLA-B37, HLA-B44, HLA-Cw6, and HLA-C clone 10. In experiments not reported here but which followed the protocol of this example, a subline of MZ2-MEL was tested, which had lost expression of HLA molecules A29, B44, and C clone 10. The subline was lysed, thus indicating that the presenting molecule should be one of A1, B37, or Cw6.

Example 2

Further studies were carried out to determine if 76/6 also produced tumor necrosis factor ("TNF") when contacted with target cells. The method used was that described by Traversari *et al.*, Immunogenetics 35: 145-152 (1992), the disclosure of which is incorporated by reference. Briefly, samples of the CTL line were combined with samples of a target cell of interest in culture medium. After 24 hours, supernatant from the cultures was removed, and then tested on TNF-sensitive WEHI cells. Cell line MZ2-MEL.43, a subclone of the MZ2-MEL cell line discussed *supra* as well as in the cited references, gave an extremely strong response, and was used in the following experiments.

Example 3

The results from Example 2 indicated that MZ2.MEL.43 presented the target antigen of interest. As such, it was used as a source of total mRNA to prepare a cDNA library.

Total RNA was isolated from the cell line. The mRNA was isolated using an oligo-dT binding kit, following well recognized

techniques. Once the mRNA was secured, it was transcribed into cDNA, via reverse transcription, using an oligo dT primer containing a NotI site, followed by second strand synthesis. The cDNA was then ligated to a BstXI adaptor, digested with NotI, size fractionated on a Sephadryl S-500 HR column, and then cloned, 5 undirectionally, into the BstXI and Not I sites of pcDNA I/Amp. The recombinant plasmid was then electroporated into DH5 α *E. coli* bacteria. A total of 1500 pools of 100 recombinant bacteria were seeded in microwells. Each contained about 100 cDNAs, because 10 nearly all bacteria contained an insert.

Each pool was amplified to saturation and plasmid DNA was extracted by alkaline lysis and potassium acetate precipitation, without phenol extraction.

Example 4

15 Following preparation of the library described in Example 3, the cDNA was transfected into eukaryotic cells. The transfections, described herein, were carried out in duplicate. Samples of COS-7 cells were seeded, at 15,000 cells/well into tissue culture flat bottom microwells, in Dulbecco's modified Eagles Medium ("DMEM") 20 supplemented with 10% fetal calf serum. The cells were incubated overnight at 37°C, medium was removed and then replaced by 50 μ l/well of DMEM medium containing 10% Nu serum, 400 μ g/ml DEAE-dextran, and 100 μ M chloroquine, plus 100 ng of the plasmids. As 25 was indicated supra, the lysis studies did not establish which HLA molecule presented the antigen. As a result, cDNA for each of the

HLA molecules which could present the antigen (A1, B37, Cw6) was used, separately, to cotransfect the cells. Specifically, one of 28 ng of the gene encoding HLA-A1, cloned into pCD-SR α , 50 ng of cDNA for HLA-B37 in pcDNA I/Amp, or 75 ng of cDNA for HLA-Cw6 in 5 pcDNA-I-Amp, using the same protocol as were used for transfection with the library.

Transfection was carried out in duplicate wells, but only 500 pools of the HLA-Cw6 transfectants could be tested in single wells.

Following four hours of incubation at 37°C, the medium was 10 removed, and replaced by 50 μ l of PBS containing 10% DMSO. This medium was removed after two minutes and replaced by 200 μ l of DMEM supplemented with 10% FCS.

Following this change in medium, COS cells were incubated for 24-48 hours at 37°C. Medium was then discarded, and 1000-3000 15 cells of CTL clone 76/6 were added, in 100 μ l of Iscove's medium containing 10% pooled human serum supplemented with 20-30 U/ml of recombinant IL-2. Supernatant was removed after 24 hours, and TNF content was determined in an assay on WEHI cells, as described by Traversari *et al.*, Immunogenetics 35: 145-152 (1992), the 20 disclosure of which is incorporated by reference.

The 1500 pools transfected with HLA-A1, and the 1500 pools transfected with HLA-B37 stimulated TNF release to a concentration of 15-20 pg/ml, or 2-6 pg/ml, respectively. Most of the HLA-Cw6 transfectants yielded 3-20 pg/ml, except for one pool, which 25 yielded more than 60 pg/ml. This pool was selected for further work.

Example 5

The bacteria of the selected pool were cloned, and 600 clones were tested. Plasmid DNA was extracted therefrom, transfected into a new sample of COS cells in the same manner as described supra, and the cells were again tested for stimulation of CTL clone 76/6. Ninety-four positive clones were found. One of these, referred to as cDNA clone 2D6, was tested further. In a comparative test COS cells were transfected with cDNA clone 2D6 and the HLA-Cw6 cDNA, HLA-Cw6 cDNA alone, or cDNA 2D6 alone. Control cell lines MZ2-MEL F⁻ and MZ2-MEL F⁺ were also used. TNF release into CTL supernatant was measured by testing it on WEHI cells, as referred to supra. The number of surviving WEHI cells was measured by optical density after incubation of the cells with MTT. Figure 2 shows that the COS cells transfected with HLA-Cw6 and cDNA-2D6, and the cell line MZ2-MEL F⁺ stimulated TNF release from CTL clone 76/6, indicating that HLA-Cw6 presented the subject TRA.

Example 6

The cDNA 2D6 was sequenced following art known techniques. A sequence search revealed that the plasmid insert showed no homology to known genes or proteins. SEQUENCE ID NO: 1 presents cDNA nucleotide information for the identified gene, referred to hereafter as "GAGE". A putative open reading frame is located at bases 51-467 of the molecule. The first two bases of this sequence are from the vector carrying the cDNA sequence, and are thus not part of the cDNA itself.

Example 7

Following sequencing of the cDNA, as per Example 6, experiments were carried out to determine if cells of normal tissues expressed the gene. To determine this, Northern blotting was carried out on tissues and tumor cell lines, as indicated below. The blotting experiments used cDNA for the complete sequence of SEQ ID NO: 1. PCR was then used to confirm the results.

Table 1. Expression of gene GAGE

Normal tissues		
5	PHA activated T cells	-
	CTL clone 82/30	-
	Liver	-
	Muscle	-
	Lung	-
	Brain	-
10	Kidney	-
	Placenta	-
	Heart	-
	Skin	-
	Testis	+
Tumor cell lines		
15	Melanoma	7/16
	Lung Carcinoma	1/6
	Sarcoma	0/1
	Thyroid medullary carcinoma	0/1
Tumor samples		
20	Melanoma	1/1

Example 8

Detailed analysis of normal tissues and tumors was carried out by applying polymerase chain reaction ("PCR") and the GAGE gene information described supra.

25 First, total RNA was taken from the particular sample, using art recognized techniques. This was used to prepare cDNA. The protocol used to make the cDNA involved combining 4 ul of reverse transcriptase buffer 5x, 1 ul of each dNTP, (10 mM), 2 ul of dithiothreitol (100 mM), 2 ul of dT-15 primer (20 μ m), 0.5 ul of

RNasin (40 units/ul), and 1 ul of MMLV reverse transcriptase (200 units/ul). Next, 6.5 ul of template RNA (1 ug/3.25 ul water, or 2 ug total template RNA) was added. The total volume of the mixture was 20 ul. This was mixed and incubated at 42°C for 60 minutes, after which it was chilled on ice. A total of 80 ul of water was then added, to 100 ul total. This mixture was stored at -20°C until used in PCR.

5 To carry out PCR, the primers

5'-AGA CGC TAC GTA GAG CCT-3'

10 (sense)

and

5'-CCA TCA GGA CCA TCT TCA-3'

(antisense)

15 SEQ ID NOS: 2 and 3, respectively, were used. The reagents included 30.5 ul water, 5 ul of PCR buffer 10x, 1 ul of each dNTP (10 uM), 2.5 ul of each primer (20 uM), and 0.5 ul of polymerizing enzyme Dynazyme (2 units/ul). The total volume was 45 ul. A total of 5 ul of cDNA was added (this corresponded to 100 ng total RNA). The mixture was combined, and layered with one drop of mineral oil. 20 The mixture was transferred to a thermocycler block, preheated to 94°C, and amplification was carried out for 30 cycles, each cycle consisting of the following:

	first denaturation:	94°C, 4 min.
	denaturation:	94°C, 1 min.
25	annealing:	55°C, 2 min.
	extension:	72°C, 3 min.
	final extension:	72°C, 15 min.

Following the cycling, 10 μ l aliquots were run on a 1.5% agarose gel, stained with ethidium bromide.

CDNA amplified using the primers set forth supra yields a 238 base pair fragment. There is no amplification of contaminating 5 genomic DNA, if present.

The results are presented in Table 2, which follows. They confirm that the only normal tissue which expresses GAGE is testis, whereas a number of tumors, including melanoma, lung, breast, larynx, pharynx, sarcoma, testicular seminoma, bladder and colon 10 express the gene. Thus, any one of these tumors can be assayed for by assaying for expression of the GAGE gene.

Tabl 2

RT-PCR analysis of the expression of gene GAGE

NORMAL TISSUES

Heart	.
Brain	.
Liver	.
Lung	.
Kidney	.
Spleen	.
Lymphocytes	.
Bone marrow	.
Skin	.
Nevus	.
Melanocytes	.
Fibroblasts	.
Prostate	.
Testis	+
Ovary	.
Breast	.
Adrenals	.
Muscle	.
Placenta	.
Umbilical Cord	.

TUMORS

	Cell lines		Tumor samples
Melanoma	40/63		45/146 (32%)
Lung cancer			
Epidermoid carcinoma		10/41	(24%)
Adenocarcinoma		4/18	
Small Cell Lung Cancer	6/23	0/2	
Breast cancer			15/146 (10%)
Head and Neck tumor			
Larynx		6/15	(40%)
Pharynx		3/13	
Sarcoma	1/4		6/18 (33%)
Testicular seminoma			6/6 (100%)
Bladder cancer			5/37 (14%)
Prostate cancer			2/20
Colon carcinoma	5/13		0/38
Renal cancer	0/6		0/45
Leukemia	3/6		0/19

Example 9

5 The identification of the nucleic acid molecule referred to in the prior examples led to the further work directed to determination of tumor rejection antigens presented by HLA-Cw6 molecules, and derived from the GAGE gene.

10 The complete cDNA of GAGE in expression vector pcDNA1/Amp was digested with restriction endonucleases NotI and SpHI, and then with exonuclease III following supplier's instruction (Erase-a-base System, Promega). This treatment generated a series of progressive deletions, starting at the 3'end.

15 The deletion products were ligated back into pcDNA1/Amp, and then electroporated into E. coli strain DH5alpha'IQ, using well known techniques. The transformants were selected with ampicillin (50 micrograms/ml).

20 Plasmid DNA was extracted from each recombinant clone and was then transfected into COS-7 cells, together with a vector which coded for HLA-Cw6. The protocols used follow the protocols described above.

25 The transfectants were then tested in the TNF release assay. This permitted separation of positive and negative clones. All the negative clones showed a deletion of the entire GAGE sequence. The smallest positive clone contained the first 170 nucleotides of SEQ ID NO: 1. The analysis of this sequence, supra, notes that the open reading frame starts at nucleotide 51. Thus, this fragment contains a sequence which encodes the first 40 amino acids of the GAGE TRAP.

Example 10

Additional experiments were then carried out to define the region encoding the TRA peptide more precisely. Polymerase chain reaction ("PCR") amplification was used to do this.

5 Two primers were synthesized. The first primer was a 22-mer complementary to a sequence within the plasmid vector pcDNA1/Amp located upstream of a BamHI site. The second primer was a 29-mer containing at the 3' end nucleotides 102-119 of SEQ ID NO: 1, and at the 5' end an extension of 11 nucleotides containing an XbaI 10 restriction site.

Following amplification, the PCR product was digested by BamHI and XbaI, and cloned into the BamHI-XbaI sites of plasmid pcDNA-3. The recombinant colonies were cotransfected into COS-7 cells with cDNA encoding HLA-Cw6, in accordance with Example 4, and a TNF 15 release assay, also as described *supra*, was carried out, using CTL 76/6.

TNF release was observed, indicating that the "minigene" was processed to a TRA. The minigene, i.e., nucleotides 1-119 of SEQ 20 ID NO: 1, the coding region of which runs from nucleotides 51-119 encoded the first 23 amino acids of the cDNA of SEQ ID NO: 1. This information served as the basis for the next set of experiments.

Example 11

Two peptides were synthesized, based upon the first 23 amino acids of SEQ ID NO: 1. These were:

Met Ser Trp Arg Gly Arg Ser Thr Tyr Arg Pro Arg Pro Arg Arg
(SEQ ID NO: 12)

and

5 Thr Tyr Arg Pro Arg Pro Arg Arg Tyr Val Glu Pro Pro Glu Met Ile
(SEQ ID NO: 13)

Each peptide was pulsed into COS-7 cells previously transfected with HLA-Cw6 cDNA, and combined with CTL 76/6 to determine if TNF release would be induced. Peptides (20 ug/ml) were added to COS-7 cells which had been transfected with the HLA-Cw6 cDNA twenty-four 10 hours previously. After incubation at 37°C for 90 minutes, medium was discarded, and 3000 CTLs were added in 100 microliters of medium, containing 25 units/ml of IL-2. Eighteen hours later, TNF content of supernatant was tested via determining toxicity on WEHI-164-13 cells. The second peptide (SEQ ID NO: 13) was found to 15 induce more than 30 pg/ml of TNF, while the first peptide (SEQ ID NO: 12), was found to induce less than 10 pg/ml of TNF. The second peptide was used for further experiments.

Example 12

20 Various peptides based upon SEQ ID NO: 13 were synthesized, and tested, some of which are presented below. To carry out these tests, ⁵¹Cr labelled LB33-EBV cells, which are HLA-Cw6 positive, were incubated with one of the following peptides:

Tyr Arg Pro Arg Pro Arg Arg Tyr
(SEQ ID NO: 4)

Thr Tyr Arg Pro Arg Pro Arg Arg Tyr
(SEQ ID NO: 5)

Tyr Arg Pro Arg Pro Arg Arg Tyr Val
(SEQ ID NO: 6)

5 Thr Tyr Arg Pro Arg Pro Arg Arg Tyr Val
(SEQ ID NO: 7)

Arg Pro Arg Pro Arg Arg Tyr Val Glu
(SEQ ID NO: 8)

10 Met Ser Trp Arg Gly Arg Ser Thr Tyr Arg Pro Arg Pro Arg Arg
(SEQ ID NO: 12)

The peptide concentration varied, as indicated in figure 3, and the ratio of CTL: LB33-EBV ("effector: target ratio"), was 10:1. ^{51}Cr release was determined after four hours of incubation at 37°C. Levels of lysis for positive ("F+", M22-MEL.3.1), and negative
15 ("F"; M22-MEL.2.2.5) control cells are indicated, in figure 3.

It was found, quite surprisingly, that the octamer of SEQ ID NO: 4 was the best peptide, and appeared to be the tumor rejection antigen. This is the first time an octamer has been reported as being involved in presentation by a human MHC molecule. There is some precedent for a murine system, as reported by Engelhard, supra, at 199, for H-2K^b and H-2K^k molecules. The nonamers of SEQ ID NO: 5 and SEQ ID NO: 6 also induced CTL lysis albeit to a less extent than the octamer of SEQ ID NO: 4.

20 In results not reported here, a second CTL was tested (CTL 82/31). This CTL was known to lyse cells presenting M22-F. It, too, lysed HLA-Cw6 positive cells following pulsing with the peptide of SEQ ID NO: 4.

Example 13

To find out whether the GAGE DNA set forth supra was unique, a cDNA library made with RNA from MZ2-MEL.43 (the same library that was used for the cloning of GAGE) was hybridized with a probe 5 derived from the GAGE cDNA. The probe was a PCR fragment of 308 base pairs between positions 20 and 328 of SEQ ID NO: 1. Twenty positive cDNAs were obtained. Six of them were entirely sequenced. They were all highly related to the GAGE sequence, but they were slightly different from it. Two of the six clones were identical 10 to each other, but all the others differed from each other. Thus, five new sequences different from but highly related to GAGE were identified. They are called GAGE-2, 3, 4, 5 and 6 (Figure 4) and are presented as SEQ ID NOS: 14-18, respectively. The fourteen other clones were partially sequenced at the 5' end and their 15 sequence corresponded to one of the six GAGE cDNAs.

The major difference between these cDNAs and GAGE-1 is the absence of a stretch of 143 bases located at position 379 to 521 of the GAGE sequence of SEQ ID NO: 1. The rest of the sequences shows mismatches only at 19 different positions, with the exception 20 of GAGE-3 whose 5' end is totally different from the other GAGE for the first 112 bases. This region of the GAGE-3 cDNA contains a long repeat and a hairpin structure.

The deduced GAGE-1 protein corresponding to a tumor rejection 25 antigen precursor is about 20 amino acids longer than the 5 other proteins, whose last seven residues also differ from the homologous residues of GAGE-1 (Figure 5). The rest of the protein sequences

show only 10 mismatches. One of these is in the region corresponding to the antigenic peptide of SEQ ID NO: 4. The sequence of the peptide is modified in GAGE-3, 4, 5 and 6 so that position 2 is now W instead of R.

5 Example 14

To assess whether the change at position 2 affected the antigenicity of the peptide, cDNA of the 6 GAGE cDNAs were individually transfected into COS cells together with the cDNA of HLA-Cw6, and the transfectants were tested for recognition by CTL 10 76/6 as described, supra. Only GAGE-1 and GAGE-2 transfected cells were recognized, showing that the modified peptide encoded by GAGE-3, 4, 5 and 6 was not antigenic in the context of this experiment. Sequence analysis of the 5' end of the 14 other clones mentioned 15 supra, showed that 7 of them contained the sequence encoding the antigenic peptide, and thus probably corresponded to either GAGE-1 or GAGE-2.

Example 15

The PCR primers used, supra to test the expression of GAGE in tumor samples do not discriminate between GAGE-1 or 2 and the four 20 other GAGE cDNAs that do not encode antigen MZ2F. A new set of primers was prepared which specifically amplifies GAGE-1 and 2, and not GAGE-3, 4, 5 and 6. These primers are:

VDE44 5'-GAC CAA GAC GCT ACG TAG-3' (SEQ ID NO: 9)
VDE24 5'-CCA TCA GGA CCA TCT TCA-3' (SEQ ID NO: 10)

These primers were used as described, supra, in a RT-PCR reaction using a polymerase enzyme in the following temperature conditions:

5

4 min at 94°C
30 cycles with
15 min at 72°C

1 min at 94°C
2 min at 56°C
3 min at 72°C

The results of this analysis are set forth in Table 3.

Table 3
Expression of GAGE genes by tumor samples and tumor cell lines

Histological type	Number of GAGE positive tumors	
	All GAGE genes*	GAGE-1 and 2**
Tumor samples		
Melanomas		
primary lesions	5/39	5/39 (13%)
metastases	47/132	36/131 (27%)
Sarcomas	6/20	6/20 (30%)
Lung carcinomas NSCLC	14/65	12/64 (19%)
Head and neck squamous cell carcinomas	13/55	10/54 (18%)
Prostatic carcinomas	2/20	2/20
Mammary carcinomas	18/162	14/162 (9%)
Bladder carcinomas		
superficial	1/20	1/20
Infiltrating	5/26	3/26
Testicular seminomas	0/6	5/6
Colorectal carcinomas	0/43	
Leukemias and lymphomas	0/25	
Renal carcinomas	0/46	
Tumor cell lines		
Melanomas	45/74	40/74 (54%)
Sarcomas	1/4	1/4
Lung carcinomas		
SCLC	7/24	7/24 (29%)
NSCLC	1/2	1/2
Mesotheliomas	3/19	3/19 (26%)
Head and neck squamous cell carcinomas	0/2	
Mammary carcinomas	1/4	0/4
Bladder carcinomas	0/3	
Colon carcinomas	5/13	5/13
Leukemias	3/6	1/6
Lymphomas	0/6	
Renal carcinomas	0/6	

* Expression of GAGE was tested by RT-PCR on total RNA with primers VDE-18 and VDE-24, detecting all GAGE genes. No PCR product was observed when these primers were assayed on DNA from MZ2-MEL.

** Expression of GAGE-1 and 2 was tested by RT-PCR on total RNA with primers VDE-44 and VDE-24, which distinguish GAGE-1 and 2 from the four other GAGE genes. No PCR product was observed when these primers were assayed on DNA from MZ2-MEL.

In further work, new primers were designed which amplified all GAGE genes, to make sure that there was no expression of any of them in normal tissues. These primers are

5 VDE43 5'-GCG GCC CGA GCA GTT CA-3' (SEQ ID NO: 11)
VDE24 5'-CCA TCA GGA CCA TCT TCA-3 (SEQ ID NO: 10)

These were used exactly as for the PCR using the VDE44 and VDE24 primers. The results are shown in Table 4. They confirm that the normal tissues are negative, except for testis.

Table 4

Expression of GAGE genes in
normal adult and fetal tissues

Adult tissues	GAGE expression*
Adrenal gland	-
Benign naevus	-
Bone marrow	-
Brain	-
Breast	-
Cerebellum	-
Colon	-
Heart	-
Kidney	-
Liver	-
Lung	-
Melanocytes	-
Muscle	-
Ovary	-
Prostate	-
Skin	-
Splenocytes	-
Stomach	-
Testis	+
Thymocytes	-
Urinal bladder	-
Uterus	-
Placenta	-
Umbilical cord	-
<u>Fetal tissues*</u>	
Fibroblasts	-
Brain	-
Liver	-
Spleen	-
Thymus	-
Testis	+

*Expression of GAGE was tested by RT-PCR amplification on total RNA with primers VDE43 and VDE24 detecting all GAGE genes (Figure 7). Absence of PCR product is indicated by - and presence by +. No PCR product was observed when these primers were assayed on DNA from MZ2-MEL.

*Fetal tissues derive from fetuses older than 20 weeks.

Example 16

In work not reported here, it had been ascertained that cytolytic T cell clone CTL 22/23 (Van der Eynde, et al., Int. J. Cancer 44: 634-640 (1989), incorporated by reference) did not recognize melanoma cell line MZ2-MEL.3.1. This melanoma cell line was reported by Van der Bruggen, et al., Eur. J. Immunol. 24: 2134-2140 (1994), to have lost expression of MHC molecules HLA-A29, HLA-B24, and HLA-CW1601. Studies were undertaken to determine if transfection with one of these MHC molecules could render the line sensitive to CTL 22/23. HLA-A29 was the first molecule tested. To do so, poly A⁺ RNA was extracted from HLA-A29⁺ cell line MZ2-MEL.43, using a commercially available extraction kit, and following the manufacturer's instructions. The mRNA was then converted to cDNA, using standard methodologies, size fractionated, and then inserted unidirectionally, into the BstI and NotI sites of plasmid pcDNA-I/Amp. The plasmids were electroporated into *E. coli* strain DH5α5'IQ, and selected with ampicillin (50 µg/ml). The bacteria were plated onto nitrocellulose filters, and duplicated. The filters were prepared, and hybridized overnight in 6xSSC/0.1% SDS/1x Denhardt's solution at 40°C, using ³²P labelled probe:

5'-ACTCCATGAGGTATTTC-3'

(SEQ ID NO: 19)

The probe is a sequence which surrounds the start codon of most HLA sequences.

The filters were washed twice, at room temperature for 5 minutes each time in 6xSSC, and twice in 6xSSC at 43°C. Positive sequences were then screened with probe:

5'-TTTCACCACATCCGTGT-3'

5 (SEQ ID NO: 20)

which had been labelled with ³²P. This sequence is specific for HLA-A29, as determined by reference to the Kabat Database of sequences and proteins of immunological interest, incorporated by reference. This database is available at the NCBI (USA), or on Web 10 Solte (Internet) WWW.NCBI.NLM.NIH.GOV. The filters were washed twice at room temperature for 5 minutes each time, at 6xSSC, followed by two washes, at 6xSSC (5 minutes per wash), at 42°C.

Example 17

Once positive HLA-A29 clones were isolated, these were 15 transfected into COS-7 using the DEAE-dextran chloroquine method set out supra. In brief, 1.5×10^4 COS-7 cells were treated with 50ng of plasmid pcDNA-I/Amp containing HLA-A29, and 100 ng of cDNA containing cDNA for one of the GAGE sequences mentioned supra, or one of the prior art MAGE or BAGE sequences in plasmid pcDNA α -I/Amp 20 or pcDSR- α , respectively. The transfectants were then incubated for 24 hours at 37°C.

The transfectants were then tested for their ability to stimulate TNF production by CTLs, using the assay explained at the end of example 4, supra.

5

Figure 7, which presents the results of this study, shows that high levels of TNF production were achieved using any of GAGE-3, 4, 5 or 6 and HLA-A29 as transfectants. GAGE-1 and GAGE-2, in contrast, do not stimulate CTL clone 22/23, thus leading to the conclusion that GAGE 3, 4, 5 and 6 are processed to an antigen or antigens presented by HLA-A29 molecules and recognized by CTL 22/23.

Example 18

10

The fact that GAGE-3, 4, 5 and 6 were processed to peptides presented by HLA-A29+ cells, while GAGE-1 and GAGE-2 were not, suggested examination of the deduced amino acid sequences for those common to GAGE 3, 4, 5 and 6 and absent from GAGE-1 and 2.

The sequence:

15 Arg Ser Thr Tyr Tyr Trp Pro Arg Pro Arg Arg Tyr Val Gln
(SEQ ID NO: 21)

was identified. The peptide was synthesized, lyophilized, and then dissolved in 1 volume DMSO, 9 volumes of 10 mM acetic acid in water. This methodology was used for the other peptides synthesized, discussed infra.

20

The peptide (SEQ ID NO: 21) was tested in a ⁵¹Cr release experiment, following the method described supra.

It was found that this peptide did provoke lysis. Successive deletions were prepared, and tested for their ability to provoke lysis, again using the ⁵¹Cr lytic assay. This work is depicted in

Figure 9. It was found that the shortest peptide to provoke lysis was

Tyr Tyr Trp Pro Arg Pro Arg Arg Tyr
(SEQ ID NO: 22), which is common to all of GAGE-3 through 6.
5 Specifically, amino acids 10-18 of GAGE-3, and amino acids 9-17 of
GAGE-4, 5 and 6 correspond to this peptide.

The members of the peptide family shown in Figure 9, and represented, e.g., by SEQ ID NOS: 21 and 22, do not accord with the data presented by Toubert, et al., "HLA-A29 Peptide Binding Motif",
10 Abstract No. 4183, Ninth International Congress of Immunology, July 23-29, 1995, San Francisco, CA, incorporated by reference. According to Toubert, et al., at the least a Phe residue is required at the third position of any peptide which binds to HLA-A29. As is shown herein, such is not the case.

15 The foregoing examples show the isolation of nucleic acid molecules which code for tumor rejection antigen precursors and tumor rejection antigens. These molecules, however, are not homologous with any of the previously disclosed MAGE and BAGE coding sequences described in the references set forth supra.
20 Hence, one aspect of the invention is an isolated nucleic acid molecule which comprises the nucleotide sequences set forth in any of SEQ ID NOS: 1-6 as well as fragments thereof, such as nucleotides 1-170, and 51-170 of SEQ ID NO: 1, or any other fragment which is processed to a tumor rejection antigen. The
25 sequences of SEQ ID NOS: 1-6 are neither MAGE nor BAGE coding sequences, as will be seen by comparing these to the sequence of

any of these genes as described in the cited references. Also a part of the invention are those nucleic acid molecules which also code for a non-MAGE and non-BAGE tumor rejection antigen precursor but which hybridize to a nucleic acid molecule containing the described nucleotide sequence of SEQ ID NO: 1, under stringent conditions. The term "stringent conditions" as used herein refers to parameters with which the art is familiar. More specifically, stringent conditions, as used herein, refers to hybridization in 1M NaCl, 1% SDS, and 10% dextran sulfate for 18 hours at 65°C. This is followed by two washes of the filter at room temperature for 5 minutes, in 2xSSC, and one wash for 30 minutes in 2xSSC, 0.1% SDS, at 65°C. There are other conditions, reagents, and so forth which can be used, which result in the same or higher degree of stringency. The skilled artisan will be familiar with such conditions, and, thus, they are not given here.

It will also be seen from the examples that the invention embraces the use of the sequences in expression vectors, as well as to transform or transfect host cells and cell lines, be these prokaryotic (e.g., *E. coli*), or eukaryotic (e.g., CHO or COS cells). The expression vectors require that the pertinent sequence, i.e., those described supra, be operably linked to a promoter. As it has been found that both of human leukocyte antigens HLA-Cw6 and HLA-A29 present tumor rejection antigens derived from these genes, the expression vector may also include a nucleic acid molecule coding for one of HLA-Cw6 or HLA-A29. In a situation where the vector contains both coding sequences, it can

be used to transfect a cell which does not normally express either one. The tumor rejection antigen precursor coding sequence may be used alone, when, e.g., the host cell already expresses one or both of HLA-Cw6 and HLA-A29. Of course, there is no limit on the particular host cell which can be used. As the vectors which contain the two coding sequences may be used in HLA-A29 or HLA-Cw6 presenting cells if desired, and the gene for tumor rejection antigen precursor can be used in host cells which do not express HLA-A29 or HLA-Cw6.

The invention also embraces so called expression kits, which allow the artisan to prepare a desired expression vector or vectors. Such expression kits include at least separate portions of each of the previously discussed coding sequences. Other components may be added, as desired, as long as the previously mentioned sequences, which are required, are included.

To distinguish the nucleic acid molecules and the TRAPs of the invention from the previously described MAGE and BAGE materials, the invention shall be referred to as the GAGE family of genes and TRAPs. Hence, whenever "GAGE" is used herein, it refers to the tumor rejection antigen precursors coded for by the previously described sequences. "GAGE coding molecule" and similar terms, are used to describe the nucleic acid molecules themselves.

The invention as described herein has a number of uses, some of which are described herein. First, the invention permits the artisan to diagnose a disorder such as melanoma, characterized by expression of the TRAP, or presentation of the tumor rejection antigen.

antigen. These methods involve determining expression of the TRAP gene, and/or TRAs derived therefrom, such as a TRA presented by HLA-Cw6 or HLA-A29. In the former situation, such determinations can be carried out via any standard nucleic acid determination assay, including the polymerase chain reaction, or assaying with labelled hybridization probes. In the latter situation, assaying with binding partners for complexes of TRA and HLA, such as antibodies, is especially preferred. An alternate method for determination is a TNF release assay, of the type described supra.

To carry out the assay, it is preferred to make sure that testis cells are not present, as these normally express GAGE. This is not essential, however, as one can routinely differentiate between testis and other cell types. Also, it is practically impossible to have testis cells present in non-testicular sample.

The isolation of the TRAP gene also makes it possible to isolate the TRAP molecule itself, especially TRAP molecules containing the amino acid sequence coded for by any of SEQ ID NOs: 2-6. These isolated molecules when presented as the TRA, or as complexes of TRA and HLA, such as HLA-Cw6 or HLA-A29, may be combined with materials such as adjuvants to produce vaccines useful in treating disorders characterized by expression of the TRAP molecule.

Exemplary adjuvants include Freund's complete and incomplete adjuvant, killed B. pertussis organisms, "BCG", or Bacille Calmette-Guerin, Al(OH)₃, muramyl dipeptide and its derivatives,

which may be emulsified in metabolizable oils, such as squalene, monophosphoryl lipid A (MPL), keyhole limpet hemocyanin (KLH), saponin extracts such as QA-7, 5 QA-19, and QA-21 (also referred to as QS-21), these having been described in U.S. Patent No. 5,057,540 to Kensil, et al., incorporated by reference, MTP-MF59, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl sulfate (DOTAP), the cationic amphiphile DOTMA, the neutral phospholipids such as DOPE, and combinations of these. This listing is by no means comprehensive, 10 and the artisan of ordinary skill will be able to augment this listing. All additional adjuvants are encompassed herein.

In addition, vaccines can be prepared from cells which present the TRA/HLA complexes on their surface, such as non-proliferative cancer cells, non-proliferative transfectants, etcetera. In all 15 cases where cells are used as a vaccine, these can be cells transfected with coding sequences for one or both of the components necessary to provide a CTL response, or be cells which express the molecules without transfection. Further, the TRAP molecule, its associated TRAs, as well as complexes of TRA and HLA, may be used 20 to produce antibodies, using standard techniques well known to the art.

When "disorder" is used herein, it refers to any pathological condition where the tumor rejection antigen precursor is expressed. An example of such a disorder is cancer, melanoma in particular. 25 Melanoma is well known as a cancer of pigment producing cells.

As indicated, supra, tumor rejection antigens, such as the one presented in SEQ ID NO: 4 are also a part of the invention. Also a part of the invention are polypeptides, such as molecules containing from 8 to 16 amino acids, where the polypeptides contain the amino acid sequence set forth in SEQ ID NO: 4. As the examples indicate, those peptides which are longer than the octamer of SEQ ID NO: 4 are processed into the tumor rejection antigen of SEQ ID NO: 4 by the HLA-Cw6 presenting cancer cells, and presented thereby. The presentation leads to lysis by cytolytic T lymphocytes present in a body fluid sample contacted to the cells presenting the complex. Similarly, the peptides longer than SEQ ID NO: 22, such as SEQ ID NO: 21, are processed to the appropriate TRA, and are presented by cancer cells, such as HLA-A29 positive cells.

Thus, another feature of the invention are peptides which are anywhere from 9 to 16 amino acids long, and comprise the sequence:

Xaa Xaa Trp Xaa Xaa Xaa Xaa Xaa Tyr

(SEQ ID NO: 23)

where Xaa is any amino acid. These peptides bind to, and/or are processed to peptides which bind to HLA-A29 molecules. The fact that these peptides are processed to the tumor rejection antigen, is indicated by the examples.

This property may be exploited in the context of other parameters in confirming diagnosis of pathological conditions, such as cancer, melanoma in particular. For example, the investigator may study antigens shed into blood or urine, observe physiological

chang s, and then confirm a diagnosis of melanoma using the CTL proliferation methodologies described herein.

On their own, peptides in accordance with the invention may be used to carry out HLA-typing assays. It is well known that when a skin graft, organ transplant, etc., is necessary one must perform HLA typing so as to minimize the possibility of graft rejection. The peptides of the invention may be used to determine whether or not an individual is HLA-Cw6 or HLA-A29 positive, so that appropriate donors may be selected. This type of assay is simple to carry out. The peptides of the invention are contacted to a sample of interest, and binding to cells in that sample indicates whether or not the individual from which the sample is taken is HLA-Cw6 or HLA-A29 positive. One may label the peptides themselves, conjugate or otherwise bind them to linkers which are labeled, immobilize them to solid phases, and so forth, so as to optimize such an assay. Other standard methodologies will be clear to the skilled artisan, and need not be presented herein.

Therapeutic approaches based upon the disclosure are premis d on a response by a subject's immune system, leading to lysis of TRA presenting cells, such as HLA-A29 or HLA-Cw6 cells. One such approach is the administration of CTLs specific to the complex to a subject with abnormal cells of the phenotype at issue. It is within the skill of the artisan to develop such CTLs in vitro. Specifically, a sample of cells, such as blood cells, are contact d to a cell presenting the complex and capable of provoking a sp cific CTL t proliferate. Th target c ll can be a

transfected, such as a COS cell of the type described supra. These transfecteds present the desired complex on their surface and, when combined with a CTL of interest, stimulate its proliferation. COS cells, such as those used herein are widely 5 available, as are other suitable host cells.

To detail the therapeutic methodology, referred to as adoptive transfer (Greenberg, J. Immunol. 136(5): 1917 (1986); Riddel et al., Science 257: 238 (7-10-92); Lynch et al., Eur. J. Immunol. 21: 10 1403-1410 (1991); Kast et al., Cell 59: 603-614 (11-17-89)), cells presenting the desired complex are combined with CTLs leading to proliferation of the CTLs specific thereto. The proliferated CTLs are then administered to a subject with a cellular abnormality 15 which is characterized by certain of the abnormal cells presenting the particular complex, where the complex contains the pertinent HLA molecule. The CTLs then lyse the abnormal cells, thereby achieving the desired therapeutic goal.

The foregoing therapy assumes that at least some of the subject's abnormal cells present the relevant HLA/TRA complex. This can be determined very easily, as the art is very familiar 20 with methods for identifying cells which present a particular HLA molecule, as well as how to identify cells expressing RNA of the pertinent sequences, in this case a GAGE sequence. Once cells presenting the relevant complex are identified via the foregoing screening methodology, they can be combined with a sample from a patient, where the sample contains CTLs. If the complex presenting 25 cells are lysed by the mixed CTL sample, then it can be assumed

that a GAGE derived, tumor rejection antigen is being presented, and the subject is an appropriate candidate for the therapeutic approaches set forth supra.

Adoptive transfer is not the only form of therapy that is available in accordance with the invention. CTLs can also be provoked in vivo, using a number of approaches. One approach, i.e., the use of non-proliferative cells expressing the complex, has been elaborated upon supra. The cells used in this approach may be those that normally express the complex, such as irradiated melanoma cells or cells transfected with one or both of the genes necessary for presentation of the complex. Chen et al., Proc. Natl. Acad. Sci. USA 88: 110-114 (January, 1991) exemplifies this approach, showing the use of transfected cells expressing HPV E7 peptides in a therapeutic regime. Various cell types may be used. Similarly, vectors carrying one or both of the genes of interest may be used. Viral or bacterial vectors are especially preferred. In these systems, the gene of interest is carried by, e.g., a Vaccinia virus or the bacteria BCG, and the materials de facto "infect" host cells. The cells which result present the complex of interest, and are recognized by autologous CTLs, which then proliferate. A similar effect can be achieved by combining the tumor rejection antigen or the precursor itself with an adjuvant to facilitate incorporation into HLA-Cw6 presenting cells which then present the HLA/peptide complex of interest. The TRAP is processed to yield the peptide partner of the HLA molecule while the TRA is presented without the need for further processing.

Other aspects of the invention will be clear to the skilled artisan and need not be repeated here.

The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

(1) GENERAL INFORMATION:

- (i) APPLICANTS: Van den Eynde, Benoit; DeBacker, Olivier; Boon-Falleur, Thierry;
- (ii) TITLE OF INVENTION: Isolated, Truncated Nucleic Acid Molecules Which Code For GAGE Tumor Rejection Antigen, The Tumor Rejection Antigen, and Uses Thereof
- (iii) NUMBER OF SEQUENCES: 18
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Felfe & Lynch
 - (B) STREET: 805 Third Avenue
 - (C) CITY: New York City
 - (D) STATE: New York
 - (E) COUNTRY: USA
 - (F) ZIP: 10022
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Diskette, 5.25 inch, 360 kb storage
 - (B) COMPUTER: IBM PS/2
 - (C) OPERATING SYSTEM: PC-DOS
 - (D) SOFTWARE: Wordperfect
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- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Hanson, Norman D.
 - (B) REGISTRATION NUMBER: 30,946
 - (C) REFERENCE/DOCKET NUMBER: LUD 5323.2
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (212) 688-9200
 - (B) TELEFAX: (212) 838-3884

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 646 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTGCCGTCCG	GAECTTTTT	CCTCTACTGA	GATTCATCTG	TGTGAAATAT	50
GAGTTGGCGA	GGAAAGATCGA	CCTATCGGCC	TAGACCAAGA	CGCTACGTAG	100
AGCCTCCTGA	AATGATTGGG	CCTATGCGGC	CCGAGCAGTT	CAGTGATGAA	150
GTGGAACCAAG	CAACACCTGA	AGAAGGGGAA	CCAGCAACTC	AACGTCAGGA	200
TCCTGCAGCT	GCTCAGGAGG	GAGAGGATGA	GGGAGGCATCT	GCAGGGTCAAG	250
GGCCGAAGCC	TGAAGCTGAT	AGCCAGGAAC	AGGGTCACCC	ACAGACTGGG	300
TGTGAGTGTG	AAGATGGTCC	TGATGGCAG	GAGATGGGACC	CGCCAAATCC	350
AGAGGAGGTG	AAAACGCCG	AAGAAGAGAT	GAGGTCTCAC	TATGTTGCC	400
AGACTGGGAT	TCTCTGGCTT	TTAATGAACA	ATTGCTTCTT	AAATCTTCC	450
CCACGGAAAC	CTTGAGTGAC	TGAAATATCA	AATGGCGAGA	GACCCTTTAG	500
TTCCTATCAT	CTGTGGCATG	TGAAGGGCAA	TCACAGTGT	AAAAGAAGAC	550
ATGCTGAAAT	GTTGCAGGCT	GCTCCTATGT	TGGAAAATTC	TTCATTGAAG	600
TTCTCCCAAT	AAAGCTTAC	AGCCTTCTGC	AAAGAAAAAA	AAAAAA	646

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AGACGCTACG TAGAGCCT

18

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CCATCAGGAC CATCTTCA

18

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Tyr Arg Pro Arg Pro Arg Arg Tyr
5

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Thr Tyr Arg Pro Arg Pro Arg Arg Tyr
5

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Tyr Arg Pro Arg Pro Arg Arg Tyr Val
5

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Thr Tyr Arg Pro Arg Pro Arg Arg Tyr Val
5 10

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amin acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Arg Pro Arg Pro Arg Arg Tyr Val Glu
5

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GACCAAGACG CTACGTAG

18

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CCATCAGGAC CATCTTCA

18

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GCGGCCGAG CAGTTCA

17

(2) INFORMATION FOR SEQ ID NO: 12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 15 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Met Ser Trp Arg Gly Arg Ser Thr Tyr Arg Pro Arg Pro Arg Arg
5 10 15

(2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Thr Tyr Arg Pro Arg Pro Arg Arg Tyr Val Glu Pro Pro Glu Met Ile
5 10 15

(2) INFORMATION FOR SEQ ID NO: 14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 538 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

ACGCCAGGGA	GCTGTGAGGC	AGTGCTGTGT	GGTTCCCTGCC	GTCCGGACTC	50
TTTTTCTCT	ACTGAGATT	ATCTGTGTGA	AATATGAGTT	GGCGAGGAAG	100
ATCGACCTAT	CGGCCTAGAC	CAAGACGCTA	CGTAGAGCCT	CCTGAAATGA	150
TTGGGCCTAT	GCGGCCCGAG	CAGTTCACTG	ATGAAGTGG	ACCAGCAACA	200
CCTGAAGAAG	GGGAACCAGC	AACTCAACGT	CAGGATCCTG	CAGCTGCTCA	250
GGAGGGAGAG	GATGAGGGAG	CATCTGCAGG	TCAAGGGCCG	AAGCCTGAAG	300
CTCATAGCCA	GGAACAGGGT	CACCCACAGA	CTGGGTGTGA	GTTGAAAGAT	350
GGTCCTGATG	GGCAGGGAGAT	GGACCCGCCA	AATCCAGAGG	AGGTGAAAAC	400
GCCTGAAGAA	GGTGAAGAAC	AATCACAGTG	TTAAAAGAAG	ACACGTTGAA	450
ATGATGCAGG	CTGCTCCTAT	GTTGAAATT	TGTTCATTA	AATTCTCCCA	500
ATAAAGCTT	ACAGCCTCT	GCAAAGAAAA	AAAAAAA		538

(2) INFORMATION FOR SEQ ID NO: 15:
(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 560 bas pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CTCATATTC	ACACAGATGA	GTTGGCGAGG	AAGATCGACC	TATTATTGGT	50
CTAGGCCAAT	AATAGGTCGA	TCTTCCTCGC	CAACTCATAT	TTCACACAGA	100
TGAATCTCAG	TAGAGGAAAA	TCGACCTATT	ATTGGCCTAG	ACCAAGGCAGC	150
TATGTACAGC	CTCCTGAAGT	GATTGGGCCT	ATGCGGCCCG	AGCAGTTCAAG	200
TGATGAAGTG	GAACCAGCAA	CACCTGAAGA	AGGGGAACCA	GCAACTCAAC	250
GTCAGGATCC	TGCAGCTGCT	CAGGAGGGAG	AGGATGAGGG	AGCATCTGCA	300
GGTCAAGGGC	CGAACGCTGA	AGCTGATAGC	CAGGAACAGG	GTCACCCACA	350
GAATGGGTGT	GAGTGTGAAG	ATGGCCTGAA	TGGGCAGGGAG	ATGGACCCGC	400
CAAATCCAGA	GGAGGTGAAA	ACGCCTGAAG	AAGGTGAAAA	GCAATCACAG	450
TGTAAAAGA	AGGCACGTTG	AAATGATGCA	GGCTGCTCCT	ATGTTGGAAA	500
TTTGTTCATT	AAAATTCTCC	CAATAAAGCT	TTACAGCCTT	CTGCAAAGAA	550
AAAAAAAAAA					560

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 540 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CGCCAGGGAG	CTGTGAGGCA	GTCGCTGTG	GTTCCCTGCCG	TCCGGACTCT	50
TTTTCTCTA	CTGAGATTCA	TCTGTGTGAA	ATATGAGTTG	GCGAGGAAGA	100
TCGACCTATT	ATTGGCCTAG	ACCAAGGCAGC	TATGTACAGC	CTCCCTGAAAT	150
GATTGGGCCT	ATGCCGCCCG	AGCAGTTCAAG	TGATGAAGTG	GAACCAGCAA	200
CACCTGAAGA	AGGGGAACCA	GCAACTCAAC	GTCAGGATCC	TGCAGCTGCT	250
CAGGAGGGAG	AGGATGAGGG	AGCATCTGCA	GGTCAAAGGGC	CGAACGCTGA	300
AGCTGATAGC	CAGGAACAGG	GTCACCCACA	GACTGGGTGT	GAGTGTGAAG	350
ATGGTCTGAA	TGGGCAGGGAG	ATGGACCCGC	CAAATCCAGA	GGAGGTGAAA	400
ACGCCTGAAG	AAGGTGAAAA	GCAATCACAG	TGTAAAAGA	AGGCACGTTG	450
AAATGATGCA	GGCTGCTCCT	ATGTTGGAAA	TTTGTTCATT	AAAATTCTCC	500
CAATAAAGCT	TTACAGCCTT	CTGCAAAAAA	AAAAAAAAAA		540

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 532 bas pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

AGCTGTGAGG	CAGTGCTGTG	TGGTTCTGC	CGTCCGGACT	CTTTTCCTC	50
TACTGAGATT	CATCTGTGTG	AAATATGAGT	TGGCGAGGAA	GATCGACCTA	100
TTATTGGCCT	AGACCAAGGC	GCTATGTACA	GCCTCTGAA	GTCGATTGGC	150
CTATGCGGCC	CGAGCAGTTC	AGTGTGAG	TGGAACCAAGC	AACACCTGAA	200
GAAGGGGAAC	CAGCAACTCA	ACGTCAGGAT	CCTGCAGCTG	CTCAGGAGGG	250
AGAGGATGAG	GGAGCATCTG	CAGGTCAAGG	GCCGAAGCCT	GAAGCTGATA	300
GCCAGGAACA	GGGTCAACCA	CAGACTGGGT	GTGAGTGTGA	AGATGGCTCT	350
GATGGGCAGG	AGATGGACCC	GCCAAATCCA	GAGGAGGTGA	AAACGCCTGA	400
AGAAGGTGAA	AAGCAATCAC	AGTGTAAAAA	GAAGGCACGT	TGAAATGATG	450
CAGGCTGCTC	CTATGTTGGA	AATTGTTCA	TTAAAATTCT	CCCAATAAAG	500
CTTTACAGCC	TTCTGCAAAG	AAAAAAAAAA	AA		532

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 539 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GCCAGGGAGC	TGTGAGGCAG	TGCTGTGTGG	TTCCTGCCGT	CCGGACTCTT	50
TTTCCTCTAC	TGAGATTCAT	CTGTGTAAA	TATGAGTTGG	CGAGGAAGAT	100
CGACCTATTA	TTGGCCTAGA	CCAAGGCCT	ATGTACAGCC	TCCTGAAGTG	150
ATTGGGCCTA	TGCGGGCCCGA	GCAGTTCACT	GATGAAGTGG	AACCAGCAAC	200
ACCTGAAGAA	GGGAAACCAAG	CAACTCAACG	TCAGGATCCT	GCAGCTGCTC	250
AGGAGGGAGA	GGATGAGGGA	GCATCTGCAG	GTCAAGGGCC	GAAGCCTGAA	300
GCTGATAGCC	AGGAACAGGG	TCACCCACAG	ACTGGGTGTG	AGTGTGAAGA	350
TGGTCCTGAT	GGGCAGGAGG	TGGACCCGCC	AAATCCAGAG	GAGGTGAAAAA	400
CGCCTGAAGA	AGGTGAAAAAG	CAATCACAGT	GTAAAAGAA	GACACGTTGA	450
AATGATGCAG	GCTGCTCCTA	TGTTGAAAT	TTGTTCATTA	AAATTCTCCC	500
AATAAAGCTT	TACAGCCTTC	TGCAAAAAAA	AAAAAAA		539

W claim:

1. An isolated peptide consisting of from 9 to 16 amino acids and containing SEQ ID NO: 23.
2. The isolated peptide of claim 1, consisting of SEQ ID NO: 23.
3. The isolated peptide of claim 1, consisting of SEQ ID NO: 21.
4. The isolated peptide of claim 1, consisting of SEQ ID NO: 22.
5. Method for determining presence of cytolytic T lymphocytes in a body fluid sample which are specific for complexes of HLA-A29 molecules and SEQ ID NO: 23, comprising contacting a sample of cells which present HLA-A29 on their surface with a polypeptide comprising SEQ ID NO: 23, under conditions favoring processing of said polypeptide to the polypeptide SEQ ID NO: 23 and binding of SEQ ID NO: 23 to said HLA-A29 molecules, contacting a body fluid sample believed to contain said cytolytic T lymphocytes to said cells presenting complexes of SEQ ID NO: 23 and HLA-A29 on their surface, and determining at least one of (i) tumor necrosis factor released by cytolytic T lymphocytes or (ii) lysis of said cells presenting said complexes, as a determination of presence of said cytolytic T lymphocytes in said sample.

6. The method of claim 5, comprising terminating release of tumor necrosis factor.
7. The method of claim 5, comprising determining lysis by determining release of radiolabelled chromium.

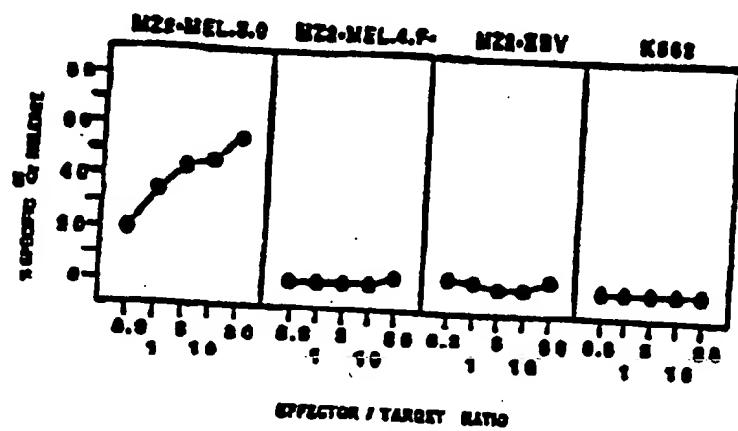


Figure 1

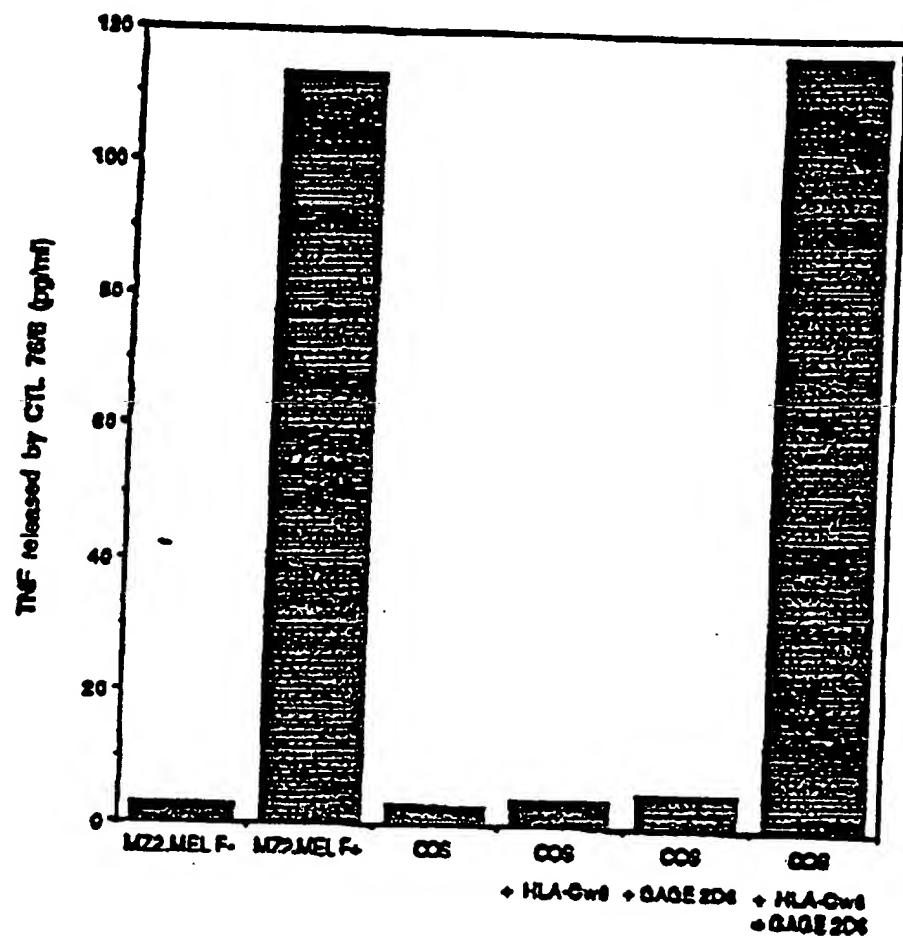


Figure 3

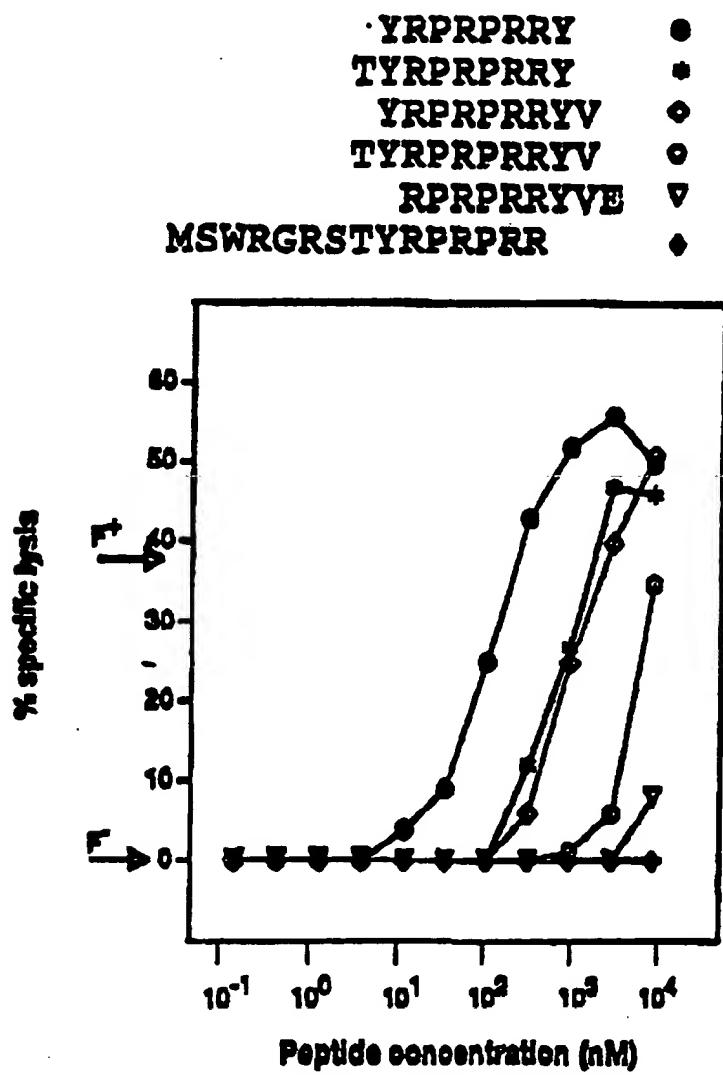
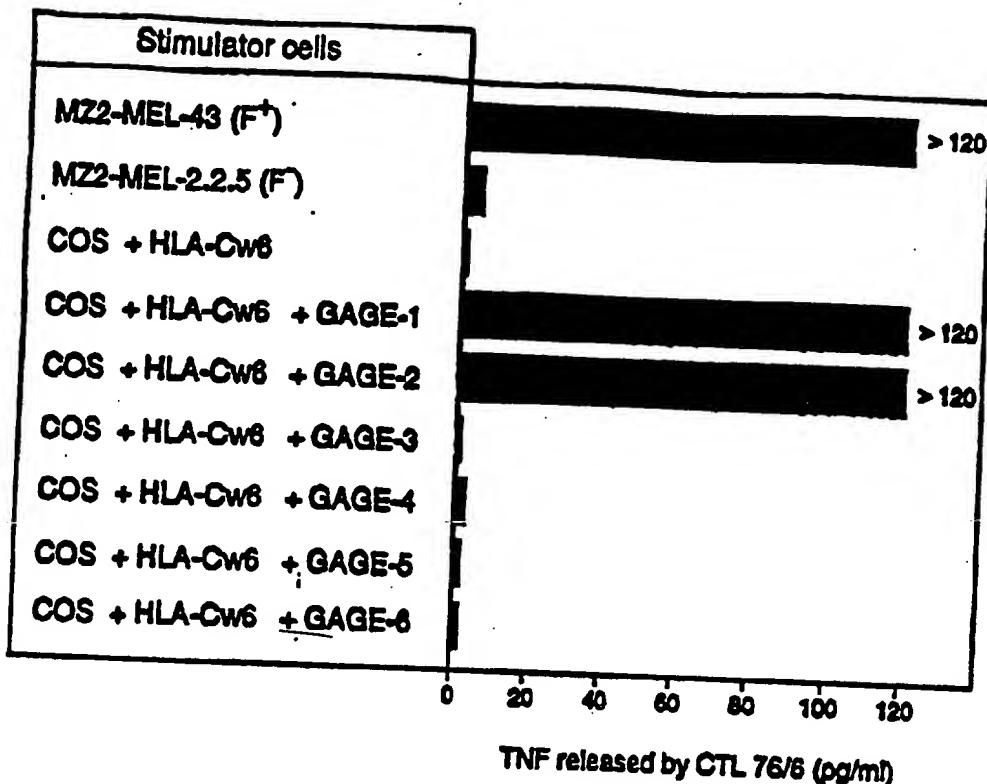


Figure 3

	antigenic peptide
GAGE-1	MS-WKGRST- [REDACTED] GPMRPEQFEDDEVAPTPPEGEPA [REDACTED] RQDPAAQEGEDEGASAGQGPKEA
GAGE-2	MS-WKGRST- [REDACTED] GPMRPEQFEDDEVAPTPPEGEPA [REDACTED] RQDPAAQEGEDEGASAGQGPKEA
GAGE-3	MS-WKGRST- [REDACTED] GPMRPEQFEDDEVAPTPPEGEPA [REDACTED] RQDPAAQEGEDEGASAGQGPKEA
GAGE-4	MS-WKGRST- [REDACTED] GPMRPEQFEDDEVAPTPPEGEPA [REDACTED] RQDPAAQEGEDEGASAGQGPKEA
GAGE-5	MS-WKGRST- [REDACTED] GPMRPEQFEDDEVAPTPPEGEPA [REDACTED] RQDPAAQEGEDEGASAGQGPKEA
GAGE-6	MS-WKGRST- [REDACTED] GPMRPEQFEDDEVAPTPPEGEPA [REDACTED] RQDPAAQEGEDEGASAGQGPKEA
GAGE-1	D5QEQGHPOTGCECECDGPDCQEDOP PNPKEVTPPEKQ5OC- [REDACTED] ILMQDQFLNLSPRP
GAGE-2	D5QEQGHPOTGCECECDGPDCQEDOP PNPKEVTPPEKQ5OC-----
GAGE-3	D5QEQGHPOTGCECECDGPDCQEDOP PNPKEVTPPEKQ5OC-----
GAGE-4	D5QEQGHPOTGCECECDGPDCQEDOP PNPKEVTPPEKQ5OC-----
GAGE-5	D5QEQGHPOTGCECECDGPDCQEDOP PNPKEVTPPEKQ5OC-----
GAGE-6	D5QEQGHPOTGCECECDGPDCQEDOP PNPKEVTPPEKQ5OC-----



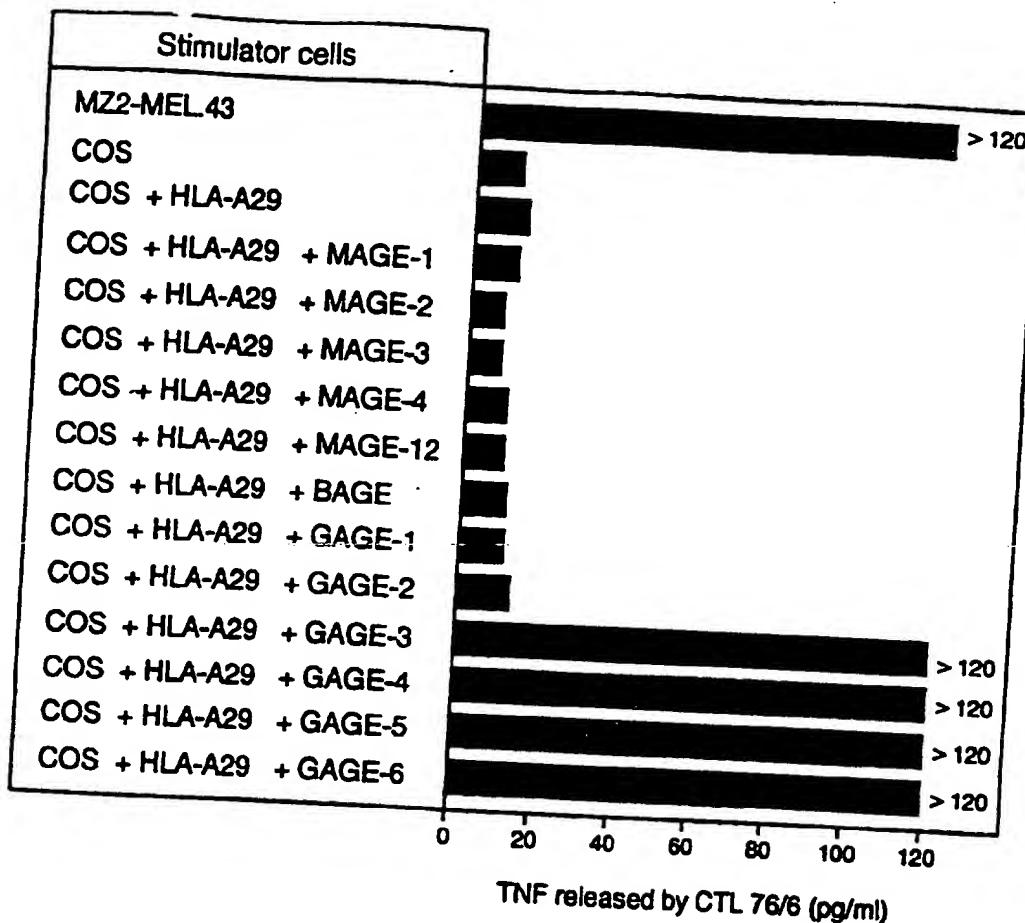


Figure 7. Stimulation of MZ2-CTL 22/23 by COS-7 cells transiently transfected with an HLA-A29 cDNA and MAGE, BAGE or GAGE cDNA. The CTL was added after 24 hours and the production of TNF was estimated 24 hours later. MZ2-MEL.43 was used as a positive control stimulator cell.

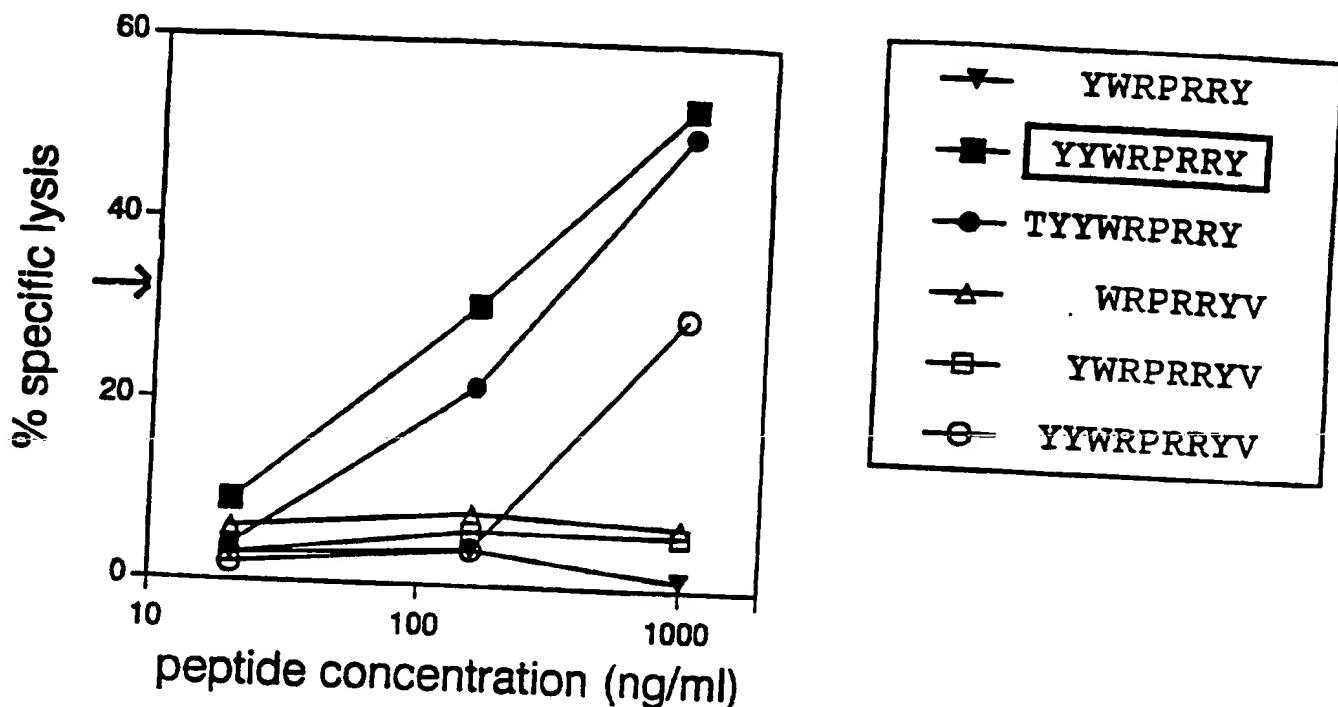


Figure 6 Lysis by MZ2-CTL 22/23 of lymphoblastoid cell line LB17-EBV incubated with GAGE-encoded peptide YYWPRPERRY. Thousand 51Cr-labelled LB17-EBV target cells were incubated in 96 well microplates in the presence of various concentrations of peptide for 15 minutes at 37°C. An equal volume containing 6000 CTL was then added. Chromium release was measured after 4 hours at 37°C. We have indicated the final concentration of peptides during the incubation of the target cells with the CTL. The arrow indicates the percentage of lysis of MZ2-MEL.43 cells.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/00381

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 7/00; C12Q 1/02; G01N 33/536, 33/574
US CL :530/300, 328, 350, 395; 435/7.1, 7.24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/300, 328, 350, 395; 435/7.1, 7.24

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
EXAMINER'S TUMOR REJECTION ANTIGEN FILES.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG (FILE=BIOCHEM). Key words: tumor rejection antigen, cytotoxic lymphocyte, GAGE, MAGE, peptid?, MHC?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Immunogenetics, volume 35, issued 1992, Traversari et al., "Transfection and expression of a gene coding for a human melanoma antigen recognized by autologous cytolytic T lymphocytes", pages 145-152, see entire article.	1-7
A	J. Exp. Med., volume 176, issued November, 1992, Traversari et al., "A Nonapeptide Encoded by Human Gene MAGE-1 is Recognized on HLA-A1 by Cytolytic T Lymphocytes Directed Against Tumor Antigen MZ2-E", pages 1453-1457, see entire article.	1-7

Further documents are listed in the continuation of Box C. See patent family annex.

• Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
• "A" document defining the general state of the art which is not considered to be part of particular relevance		
• "E" earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
• "L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
• "O" document referring to an oral disclosure, use, exhibition or other means		
• "P" document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 APRIL 1996	15 MAY 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer <i>Thomas M. Cunningham</i> THOMAS M. CUNNINGHAM
Faxsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/00381

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Science, volume 254, issued 13 December 1991, van der Bruggen et al., "A Gene Encoding an Antigen Recognized by Cytolytic T Lymphocytes on a Human Melanoma", pages 1643-1647, see entire article.	1-7